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Detection of Neighboring Weeds Alters Soybean Seedling Roots and Nodulation

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Crop and weed competition studies rarely determine how plant-to-plant interactions alter the structure and physiology of crop roots. Soybean has the ability to detect neighboring weeds and to alter growth patterns including the allocation of resources to root growth. In this study, we hypothesized that low red : far red light ratio (R : FR) reflected from aboveground vegetative tissue of neighboring weeds would alter soybean root morphology and reduce root biomass and nodule number. All experiments were conducted under controlled conditions in which resources of light, water, and nutrients were nonlimiting. Low R : FR reflected from aboveground neighboring weeds reduced soybean seedling root length, surface area, and volume, including the number of nodules per plant. An accumulation of H_2O_2, an increase in malondialdehyde (MDA) content, a reduction in flavonoid content, and a decrease in 1,1-diphenyl-2-picrylhydrazyl (DPPH)–radicle scavenging activity were observed. The reduction in flavonoid content was accompanied by a decrease in the transcription of *Gm*IFS and *Gm*N93 and an increase in transcript levels of several antioxidant genes. These molecular and physiological changes may have a physiological cost to the soybean plant, which may limit the plant's ability to respond to subsequent abiotic and biotic stresses that will occur under field conditions.

Nomenclature: Soybean [*Glycine max* (L.) Merr.]
Key words: Antioxidants, flavonoid content, gene expression, hydrogen peroxide, light quality, plant competition, red : far red.

Crop–weed competition studies rarely determine how plant-to-plant interactions alter the structure and physiology of crop roots. Despite being immobile, plants have the ability to detect changes in their surrounding environment and rapidly integrate this information into alternative patterns of growth. It is well established that plants are able to detect the presence of neighbors through changes in light quality signals, specifically a lowered red : far red light ratio (R : FR) (Casal et al. 1987; Kasperbauer 1987). Detection of low R : FR by the phytochrome system enables the plant to respond morphologically to pending low light conditions that may result from the rapid growth of neighboring plants (Ballaré et al. 1987; Ballaré and Casal 2000). Such morphological changes, commonly referred to as shade-avoidance traits, include stem elongation, reduction in tillering, increased apical dominance, and altered leaf shape and leaf-area distribution (Ballaré et al. 1990; Casal et al. 1987; Kasperbauer and Karlen 1994; Green-Tracewicz et al. 2011, 2012; Yang et al. 2014). The ability to detect changes in aboveground light quality signals and transfer this information to roots is an essential survival strategy to ensure optimal fitness under competitive conditions.

Changes in light quality such as the R : FR may not only signal pending plant competition but also trigger molecular, physiological, and morphological changes that precondition the crop to be more susceptible to subsequent abiotic and biotic stresses, including direct competition for resources (Page et al. 2009, 2011). Affirmation of this central role of low R : FR reflected from neighboring weeds to trigger molecular and physiological changes in maize (*Zea mays* L.) was reported by Afifi and Swanton (2012). In this study, conducted under conditions of nonlimiting resources, the detection by phytochrome of low R : FR signals reflected from both biological (weed seedlings) and nonbiological (commercial filter) sources triggered typical morphological shade-avoidance responses, as well as an upregulation of ethylene biosynthesis genes, stimulation of an auxin transport gene, a reduction in anthocyanin content, an enhancement of lignin synthesis, an accumulation of H_2O_2 in the first leaf and crown root tissues, and the closure of stomata in the first leaf of a corn seedling. The authors suggested that these physiological changes may result in a physiological cost that may contribute to the rapid loss in yield observed in weed competition studies conducted under field conditions.

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In addition to the central role of low R : FR, other variables, such as reduced photosynthetically active radiation (PAR), blue light and organic volatile compounds, such as ethylene, have also been implicated as important signals detected from neighboring plants, which can induce typical shade-avoidance phenotypic responses. Under conditions of canopy closure PAR will decrease. Low PAR has been reported to cause hyponasty and stem elongation (Ballaré et al. 1991; Pierik and de Wit 2013). Low blue light is capable of causing phenotypic responses similar to low R : FR such as hypocotyl elongation (Ballaré et al. 1991), internode and petiole elongation and hyponastic leaf movement (Pierik et al. 2004). Blue light is mainly absorbed by leaves, with potentially small amounts reflected. The role of blue light becomes most important as photosynthetic light diminishes, such as when crop canopy closure occurs, or when plants are grown under dense vegetation or at the end of the daily photosynthetic period (sunset). Under these conditions, both photosynthetic photon flux density (PPFD) and red light are also depleted. Green light has also been found to affect physiological and morphological development in plants (Folta and Maruhnich 2007); however, the effect of green light is thought to be restricted to low light conditions (Wang et al. 2013). Under a dense canopy created by high density tobacco planting, Pierik et al. (2004) clarified that within dense canopies, ethylene concentrations can increase to levels capable of inducing typical shade-avoidance morphological traits. Under experimental conditions of nonlimiting resources (Afifi and Swanton et al. 2012; Green-Tracewicz et al. 2012; Liu et al. 2009; Page et al. 2009) where there was no limitation to PPFD, no plant-canopy–inducing shade conditions and appropriate levels of water and nutrients, the role of PAR, blue light, and ethylene signals may have limited involvement. In experiments designed specifically to explore the mechanism of nonlimiting resource plant competition, changes in the R : FR signal reflected from neighboring plants have provided direct and consistent evidence of the central role that this signal plays in modifying plant physiology and morphology.

Exposure to low R : FR is well known to reduce root biomass (Kasperbauer and Karlen 1986; Liu et al. 2009; Page et al. 2009; Pecháčková 1999; Skálová and Vosátka 1998). Wheat (*Triticum aestivum* L.) seedlings exposed to FR light developed fewer roots and had a higher shoot : root than unshaded plants (Kasperbauer and Karlen, 1994). Page et al. (2009) found that maize root biomass was reduced progressively as the duration of exposure to neighboring weeds increased. Afifi and Swanton (2011) reported that maize roots originating from seed or stem tissue differ in their response to changes in the R : FR reflected from neighboring weeds. Limited research has been conducted to determine how R : FR light signals reflected from the vegetative tissue of neighboring weeds affect soybean root physiology and morphology.

Low R : FR reflected from aboveground neighboring weeds reduced soybean total root biomass by 36% by the V2 stage of soybean development (Green-Tracewicz et al. 2011). In addition, a recent field intercropping study conducted by Yang et al. (2014) examined the effects of light quantity and R : FR on the growth of soybean seedlings growing in a relay strip intercropping system. In agreement with previously reported results, researchers found that root length, total root biomass and root : shoot of soybean were decreased significantly by the combined effects of altered light quality and reduced light quanta in a field environment. Neither of these studies, however, determined the effect of low R : FR on soybean nodulation.

A limited number of studies have been published on the effects of low R : FR on soybean root physiology and nodulation. In controlled environmental studies, Kasperbauer et al. (1984) reported that soybean plants receiving low R : FR partitioned more photosynthate to stems and less to roots, resulting in smaller roots and a lower number of nodules when compared to plants receiving higher levels of red light. Hunt et al. (1989) reported that soybean nodulation was not affected by changes in R : FR reflected from insulation panels painted red, white, or black or from bare or oat (*Avena sativa* L.) straw residue covered soils. In an additional experiment designed to determine the effect of two strains of *Bradyrhizobium japonicum* and R : FR on soybean nodulation, Hunt et al. (1990) found that even small changes in reflected R : FR light could affect soybean nodulation, and this effect varied with inoculated strain of *B. japonicum.* None of these studies, however, explored the physiological mechanisms triggered by changes in the R : FR light signals that led to the reduction in nodule number, nor were these studies conducted within the context of a mechanistic approach to understanding weed competition.

Understanding the physiological mechanisms that occur in soybean roots in response to the presence of neighboring weeds is critical to our understanding of nonlimiting resource competition. Therefore, we hypothesized that the low R : FR signal reflected from the vegetative tissue of neighboring weeds, and perceived by the phytochrome system, will influence negatively soybean root structure and biomass, and reduce nodule number.

Materials and Methods

Experiment I. Plant Materials and Growth Condition. In order to study the effect of aboveground neighboring weeds on soybean root morphology, soybean seeds of the University of Guelph, OAC Wallace variety, were selected for this study. Soybean seeds were planted 2 cm deep into Turface MVP, a clay baked medium (Profile Products LLC, Buffalo Grove, IL) in clean, 8-cm-diameter, 10-cm-tall, 355-ml plastic cups (one seed per cup) (Dart Container Corporation, Mason, MI). These cups were then positioned within 8-cm-diameter, 18-cm-tall pots (1-L natural cylinder modified to 18 cm; Consolidated Bottle, Toronto, ON, Canada). These cylindrical pots were further centered within 25-cm-diameter, 19-cm-tall 6-L pots (Airlite Plastics Company, Omaha, NE). The area surrounding the cylindrical pot was filled with Turface, a 100% backed calcined clay growth media with grain size between 2.5 and 3.5 mm (Turface MVP; Profile Products LLC, Buffalo Grove, IL; herein referred to as weed-free), or to established perennial ryegrass (*Lolium perenne* L.) as the model weed species (herein referred to as weedy) as described in Green-Tracewicz et al. (2011). This potting arrangement isolated the roots of the soybean seedlings from those of the perennial ryegrass, thereby eliminating the effects of direct root competition for water, nutrients, or any allelopathic effect (Green-Tracewicz et al. 2011).

Sixty-four weedy and 64 weed-free pots were placed within the same growth cabinet (Conviron, model PGW36, Controlled Environments Ltd., Winnipeg, MB). Twenty-four of the pots for each treatment were positioned as border rows around the outside of the chamber. Growth conditions were set to a 23 : 15 C day : night temperature and a 16 : 8 h (day : night) photoperiod, at 60 to 65% humidity. Irradiance was supplied by a sliding bank of Sylvania F48T12/CW/VHO 115 W Hg tubes and 40 W tungsten bulbs delivering a total of 550 µmol m^{-2} s^{-1} PPFD. A point quantum radiometer (LI-190SA, LI-COR Biosciences Lincoln, NE) with a cosine-corrected sensor on a fiber-optic cable was used to measure incoming PPFD at the top of the soybean seedlings.

The R : FR of the light reflected from the ryegrass canopy and the Turface MVP media was measured with the use of a R : FR sensor (SKR 110, 660/730 nm, Skye Instruments Ltd., Llandrindod Wells, Powys, UK). This measurement was recorded immediately after the soybean seeds were planted and at random intervals during the growth period. The R : FR of incoming irradiance did not differ between treatments (approximate values of 2.5 to 2.8). The R : FR reflected from the surface of the ryegrass and Turface MVP was determined by positioning the sensor downward, 5 cm above either surface at four different points within each treatment. The R : FR in the weed-free treatment, containing only Turface MVP, was (R : FR ± SE) 1.33 ± 0.15 and 0.67 ± 0.16 in the weedy treatment.

The soybean seedlings were watered daily, and fertilized twice per week with the complete nutrient solution described by Tollenaar (1989). The ryegrass was watered every other day, and fertilized with the same nutrient solution, two times per week. In order to minimize shading potential, the ryegrass was manually clipped as required to remain below the soybean unifoliate leaves. A total of five replicates (in time) were completed in the same growth cabinet for this experiment. The weedy and weed-free sides of the chamber were randomly assigned for each replicate.

Shoot and Root Morphology Measurements. Eight plants from each treatment were selected randomly for harvest at emergence (VE), cotyledon (VC), unifoliate (V1), first trifoliate (V2), and second trifoliate (V3) stages of development. Shoot height and fresh root morphology (including root volume, surface area, diameter, length, and number of root tips) were measured and recorded for both weedy and weed-free treatments. At each harvest, soybean roots were washed with tap water and cut under the hypocotyl area from the shoot. Shoot height was recorded in order to confirm a shade-avoidance response (i.e., stem elongation). The soybean shoot from each seedling was dried at 80 C to a constant weight.

Fresh root morphology was analyzed with the use of WinRhizo software (Regent Instruments Inc., Sainte-Foy, QC, Canada). Each individual root system was spread as evenly as possible on the tray and imaged at a medium resolution (200 to 400 dpi) with the use of an Epson Expression 10000XL scanner (Epson America Inc., Long Beach, CA). Upon completion of the analysis, each root system was dried at 80 C to a constant weight. Once dried, the individual weight of each root and shoot from each seedling was recorded for further analyses.

Experiment II. Plant Materials and Growth Condition. The objective of this experiment was to study the effect of aboveground neighboring weeds on soybean seedling root nodule number and root physiological responses. OAC Wallace soybean was selected for this study. OAC Wallace soybean seedlings were grown in a 1 : 1 mixture of Turface MVP and grade 2a vermiculite (Therm-O-Rock East Inc., New Eagle, PA) and inoculated with HiStick N/T (Becker Underwood [BASF], Saskatoon, SK, Canada) commercial inoculant peat. Plants were grown and exposed to weed-free and weedy treatments as described above.

Soybean Root Nodule Number and Nutritional Analysis. All soybean seedlings ($N = 100$) were harvested at the unifoliate stage of development. Roots were washed thoroughly with tap water after which the number of root nodules per plant was counted and recorded. Root and shoot of each plant was dried to a constant weight at 80 C and sent to the University of Guelph's Laboratory Services and analyzed for total carbon and total nitrogen content with the use of standard combustion methodology.

Analysis of H_2O_2 Concentration. Hydrogen peroxide in the ground root tissues was estimated according to the protocol reported by Patterson et al. (1984). One hundred milligrams of frozen ground tissue was homogenized in 200 µl cold acetone. After centrifuging for 5 min at 10,000 *g*, the supernatant was mixed with 20 µl of titanium reagent (2% $TiC1_2$ in conc. HCl). The Ti-H_2O_2 complex was precipitated by adding 40 µl of 15-M ammonia solution. This solution was centrifuged as described above; then the pellet was washed with cold acetone two times and then dissolved in 1 ml of 4 N H_2SO_4. The absorbance of the solution was measured at 410 nm against blanks that had been prepared similarly but without plant tissue.

Analysis of Lipid Peroxidation. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids found in phospholipids and is responsible for cell membrane damage (Halliwell and Gutteridge 1984). Lipid peroxidation was measured by determining the MDA content of the ground root tissues with the use of a thiobarbituric acid (TBA) reaction as described by Hara et al. (2003). One hundred milligrams of frozen, ground, unifoliate soybean seedling root tissues were homogenized in 1 ml of 5-mM potassium phosphate buffer (pH 7). After centrifuging at 4 C for 15 min at 12,000 *g*, an aliquot of the supernatant (900 µl) was mixed with 600 µl of TBA solution containing 10% (w/v) sodium dodecyl sulphate (SDS), 20% (w/v) acetic acid, 0.8% (w/v) aqueous TBA, and deionized water. The control reaction was a mixture of 900 µl of 5-mM KP buffer and 600 µl of the TBA solution. These mixtures were incubated at 98 C for 60 min, and then cooled to room temperature. Mixtures were centrifuged at 12,000 *g* for 15 min at room temperature. The absorbance of the mixture was measured at 535 and 600 nm. The MDA content was calculated from the subtracted absorbance (A535 to A600) with the use of a molecular extinction coefficient of (1.56×10^5 M^{-1} cm^{-1}).

Analysis of Flavonoid Content. Total flavonoid contents of root tissue were measured with the aluminum chloride colorimetric assay using the protocol reported by Patel et al. (2010). One milliliter of 100% ethanol was added to 0.1 g of ground, frozen, unifoliate soybean seedling root tissue. The mixture was vortexed on high for 1 min, three times. Samples were then put into the centrifuge at 12,000 rpm for 15 min. The supernatant was transferred to a fresh 1.5-ml Eppendorf tube and stored in the dark, at 4 C, for further analysis.

In a fresh 2 ml Eppendorf tube, 800 µl of distilled water, 200 µl of the above ethanolic extract, and 60 µl of 5% $NaNO_2$ were added. The mixture was briefly vortexed, and incubated at room temperature for 5 min. After the addition of 60 µl of 10% $AlCl_3$, the mixture was incubated at room temperature for an additional 6 min. Before transferring 1 ml of the mixture to a fresh cuvette, 400 µl of 1-M NaOH and 480 µl of distilled water were added, and the sample was vortexed. The absorbance of the reaction mixture was measured at 510 nm. Total flavonoid content of the samples was expressed as a percentage of quercetin equivalent per gram fresh weight.

Analysis of 1,1-Diphenyl-2-Picrylhydrazyl (DPPH)–Radicle Scavenging Activity. The antioxidant capacity of the sample extracts was tested by the evaluation of the free-radicle—scavenging effect on the DPPH radicle, according to the method of Abe et al. (1998). Briefly, 0.1 g of frozen ground tissue from each seedling

Table 1. Primer sequences used in performing quantitative real-time polymerase chain reaction.

Primer name	Forward primer sequence	Reverse primer sequence	Accession number
*Gm*MnSOD	5′-GGTCTGGACAAAGAGTTGAAGA-3′	5′-GCATGCTCCCAAACATCAATAC-3′	EF587264
*Gm*GPX	5′-GACAAAGCTGCTCCACTGTA-3′	5′-GATCAACCACATTTCCCTCTTTATC-3′	Glyma05g37900.3
*Gm*APX3	5′-CCCTGGACCTCTAATCCTCTTA-3′	5′-CTTGTCAGAAGGTAGCTGAAGG-3′	U56634.1
*Gm*CATl4	5′-ATTGGAGGAAGAGGCCATTAAG-3′	5′-CGAACAGTTTCCACTCAGGATAG-3′	NM_001250642.1
*Gm*CuZnSOD	5′-CTGGACCAAACTCCATCATAGG-3′	5′-TACTCTGCCACCAGCATTTC-3′	NM_001248369
*Gm*FeSOD	5′-GCTTGATGGGAAGTCACTAGAA-3′	5′-CATGCACTCCCAGAAGAAGT-3′	M64267
*Gm*N93	5′-GCAGTTGTTGCCAGTGTTG-3′	5′-GAGAGCTTGAGCTGTGTGATT-3′	D13506
*Gm*IFS	5′-GGAGAGAACGAGAAGAACACAA -3′	5′-TTGCACCTTCCACTTCCTTAG-3′	FJ770473.1

tissue was extracted with 1 ml of 99.5% methanol. The extract was then centrifuged at 12,000 *g* for 15 min. An aliquot of (100 µl) of the methanolic extract was mixed with 400 µl of absolute ethanol, 250 µl of 0.5-mM DPPH, and 500 µl of 100-mM acetate buffer (pH 5.5). The mixture was vortex mixed and kept in the dark for 30 min. The absorbance of the solution was measured at 517 nm against blanks of DPPH solution that had been prepared similarly but without plant tissue. Results were expressed as the percentage of inhibition of the DPPH radicle, which was calculated according to according to the following equation:

$$\%\ \text{inhibiton of DPPH} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100,$$

where A_{Control} is the absorbance reading of DPPH in the solution without extracts and A_{Sample} is the absorbance reading of DPPH within the sample solution.

Analysis of Gene Expression with the Use of Quantitative Real-Time PCR (QRT-PCR). QRT-PCR was conducted to test the transcript response of *Gm*MnSOD, *Gm*GPX, *Gm*APX2, *Gm*CAT4, *Gm* CuZnSOD, *Gm*FeSOD, *Gm*N93, and GmIFS genes to the presence of neighboring weeds. Total RNA from each treatment was isolated from the different seedling tissues with the use of TRIzol Reagent (Life Technologies Inc., Burlington, ON, Canada). To eliminate any residual genomic DNA, total RNA was treated with RQ1 RNase-free DNase (Promega, Fitchburg, WI). The first strand cDNA was synthesized from total RNA by using the Reverse Transcription System Kit (Quanta, MD). Primer Express 2.0 software (Applied Biosystems, CA) was used to design the primers for the target genes (see description of primer sequences in Table 1). Results were standardized to the housekeeping gene *Gm*Ubi (Matthews et al. 2014). As described in Livak and Schmittgen (2001), relative quantification (RQ) values for each target gene relative to the internal control tubulin was calculated by the $2^{-\Delta\Delta C}$ T method.

Statistical Analysis

Morphology Experiments. Experiments were designed as a randomized complete block. Statistical analyses were performed in SAS v9.3 (SAS Institute, Cary, NC) with a Type I error rate set at the 5% significance level. Soybean seedlings harvested at VE, VC, V1, V2, and V3 developmental stages were analyzed with the use of repeated-measures analysis of variance (ANOVA) carried out with the use of PROC MIXED, generating means and standard errors for each treatment at each stage. No transformations were required for analysis. In this experiment, replications were defined as growth cabinet environments in time and were combined for analysis. Replications were partitioned as random effects. Fixed effects included treatment, sampling time, and the interaction between these effects. Residual analysis was performed to test for the assumptions of ANOVA. Residuals and predicted values were plotted to ensure the homogeneity of variance, and independence of errors. The mean residuals of the morphological parameters (including height, root surface area, root volume, root diameter, and number of root tips, as well as the normality of the error distribution) were tested with PROC UNIVARIATE. The Shapiro-Wilk statistic was used to test the assumption of normality. The significance of the random and fixed effects was tested with the use of an *F* test.

Molecular and Physiology Experiments. Experiments were designed as a randomized complete block. Statistical analyses were performed in SAS V9.3 (SAS Institute, Cary, NC, USA) with a Type I error rate set at the 5% significance level. Soybean

Table 2. The effect of aboveground neighboring weeds on shoot height (cm), shoot dry weight (g), and root dry weight (g) measured from soybean emergence until the second-trifoliate stage of soybean development. Data are means (± SE). Type I error rate set at 5% significance level.[a]

		Shoot height			Shoot dry weight			Root dry weight		
		Treatment			Treatment			Treatment		
DAP	Soybean stage	Weed free	Weedy	P value	Weed free	Weedy	P value	Weed free	Weedy	P value
		cm			g			g		
6	VE	2.4 (0.44)	2.7 (0.44)	0.59	0.14 (0.010)	0.14 (0.010)	0.94	0.02 (0.008)	0.02 (0.008)	0.80
8	VC	4.5 (0.42)	5.3 (0.42)	0.03	0.13 (0.010)	0.12 (0.010)	0.33	0.03 (0.007)	0.02 (0.007)	0.14
12	V1	7.4 (0.41)	9.4 (0.42)	< 0.0001	0.16 (0.010)	0.16 (0.010)	0.96	0.07 (0.007)	0.05 (0.007)	0.06
17	V2	10.8 (0.44)	15.1 (0.44)	< 0.0001	0.30 (0.010)	0.33 (0.010)	0.18	0.13 (0.008)	0.12 (0.008)	0.25
21	V3	14.5 (0.43)	19.5 (0.45)	< 0.0001	0.51 (0.010)	0.50 (0.010)	0.13	0.20 (0.007)	0.17 (0.007)	0.0008

[a] Abbreviations: DAP, days after planting; VE, emergence; VC, cotyledon; V1, unifoliate; V2, first trifoliate; V3, second trifoliate.

seedlings harvested at the unifoliate stage of development were analyzed with the use of a one-way analysis of variance (ANOVA) carried out with PROC MIXED, generating means and standard errors for each treatment. No transformations were required for analysis. Growth cabinets were partitioned as random effects. Treatment was a fixed effect. Residual analysis was performed to test for the assumptions of ANOVA. Residuals and predicted values were plotted to ensure the homogeneity of variance, and independence of errors. The mean residuals of nodule number, DPPH-radicle scavenging activity, H_2O_2 content, lipid peroxidation, and flavonoid content, as well as the normality of the error distribution, were tested with PROC UNIVARIATE. The Shapiro-Wilk statistic was used to test the assumption of normality. The significance of the random and fixed effects was tested with the use of an *F* test.

Results and Discussion

Soybean Seedlings Expressed Shade Avoidance Characteristics in Response to the Presence of Aboveground Neighboring Weeds. This study was conducted to ensure that soybean seedlings grown under the described conditions would express shade avoidance characteristics in response to the proximity of neighboring weeds. As expected, plant height of soybean seedlings increased when exposed to neighboring weed seedlings. Seedlings were first examined at emergence, and although there was an increase in shoot height at this stage, it was not significant; however, from the cotyledon to second trifoliate stages, differences were apparent (Table 2). For example, at the cotyledon stage, shoot height increased from 4.5 cm ± 0.42 for weed-free seedlings to 5.3 cm ± 0.42 for weedy seedlings. The longer seedlings were exposed to low R : FR as a consequence of neighboring weeds, the greater the increase in shoot height. By the second trifoliate stage, shoot height in weedy plants was 19.5 cm ± 0.45, as compared to 14.5 cm ± 0.43 in weed-free plants, a difference of approximately 5 cm. Although plants exposed to neighboring weeds displayed the classic shade-avoidance response of increased shoot height, this was not reflected in measurements of shoot dry weight, regardless of developmental stage.

Although shoot dry weights did not differ between treatments, differences were found with root dry weight and the root : shoot ratio. Despite a difference of approximately 5 cm in shoot height at the second trifoliate stage of development for plants growing in the weedy treatment compared to the weed-free treatment, this height difference was not indicative of an increase in shoot dry weight. Shoots of seedlings exposed to neighboring weeds weighed 0.50 g ± 0.010, compared to 0.51 g ± 0.010 for the weed-free plants at the second trifoliate leaf stage (Table 2). Root dry weight, however, was reduced by the presence of weeds sampled at the second trifoliate leaf stage. At this stage, root dry weight per plant was 0.17 g ± 0.007, compared to 0.20 g ± 0.007 in the weed-free plants. Differences in root : shoot between treatments were dependent on stage of soybean development, and were detected only at the cotyledon and unifoliate stages of soybean development (Table 3). These changes in root dynamics were also reflected in additional root parameters.

Low R : FR reflected from aboveground neighboring weeds reduced soybean seedling root length, surface area, and volume. Total root length was decreased by the presence of aboveground neighboring weeds only at the second trifoliate stage of development, compared to soybean seedlings grown in a weed-free environment (Table 3). At the

Table 3. The effect of aboveground neighboring weeds on root: shoot, total root length (mm), and root surface area (cm^2) measured from soybean emergence until the second-trifoliate stage of soybean development. Data are means (± SE). Type I error rate set at 5% significance level.

		Root : shoot			Total root length			Root surface area		
		Treatment			Treatment			Treatment		
DAP	Soybean stage	Weed free	Weedy	P value	Weed free	Weedy	P value	Weed free	Weedy	P value
					mm			cm^2		
6	VE	0.16 (0.050)	0.11 (0.053)	0.5	5 (26.2)	7 (26.8)	0.93	2.7 (5.48)	2.9 (5.61)	0.97
8	VC	0.30 (0.045)	0.18 (0.045)	0.05	80 (24.7)	74 (24.8)	0.83	19.3 (5.20)	18.2 (5.23)	0.81
12	V1	0.44 (0.045)	0.31 (0.046)	0.04	248 (24.7)	224 (25.0)	0.30	53.8 (5.20)	49.6 (5.26)	0.38
17	V2	0.43 (0.051)	0.37 (0.050)	0.39	702 (26.4)	662 (26.1)	0.13	134.4 (5.52)	122.0 (5.48)	0.02
21	V3	0.39 (0.045)	0.35 (0.045)	0.54	1063 (24.8)	975 (24.8)	0.0002	187.5 (5.23)	163.4 (5.23)	< 0.0001

[a] Abbreviations: DAP, days after planting; VE, emergence; VC, cotyledon; V1, unifoliate; V2, first trifoliate; V3, second trifoliate.

second trifoliate leaf stage, total root length of seedlings exposed to aboveground neighboring weeds was 975 mm ± 24.8, compared to those kept weed-free (1,063 mm ± 24.8). Accompanying this reduction in total root length was a decrease in root surface area and volume, detected at the first and second trifoliate stages of soybean development. At the first trifoliate leaf stage, root surface area was reduced from 134.4 cm^2 ± 5.52 in weed-free plants to 122.0 cm^2 ± 5.48 in weedy plants (Table 3). Similar results were observed in root volume (Table 4). By the first trifoliate stage, the presence of aboveground neighboring weeds reduced the root volume of soybean seedlings from 2.05 cm^3 ± 0.095 in the weed-free treatment to 1.80 cm^3 ± 0.094. By the second trifoliate, root volume was further decreased from 2.65 cm^3 ± 0.090 in weed-free plants to 2.20 cm^3 ± 0.090.

Despite observed changes in root volume, the average root diameter and the number of root tips were not affected by the presence of aboveground neighboring weeds (Table 4). At emergence, for example, the average root diameter was 1.05 mm ± 0.022, compared to 1.08 mm ± 0.021 in weed-free plants. No differences were detected by the second trifoliate stage of soybean development. The average number of root tips followed a similar trend, as no differences were observed between treatments at any stage of soybean growth.

Soybean Root Nodules per Plant Were Reduced by the Presence of Aboveground Neighboring Weeds. Low R : FR reflected from neighboring weeds decreased the number of root nodules per plant when sampled at the unifoliate stage of soybean development. Nodule numbers per plant were reduced from an average of 28 ± 2.7 in the weed-free treatment, compared to 20 ± 2.8 nodules per plant when grown in the presence of weeds, a difference of approximately 29% (Figure 1). When expressed, however, on a root dry weight basis in order to account for the observed reduction in total root biomass which occurred in the weedy treatment, nodule number was 260 ± 25.2 nodules g^{-1} of dry root in the weed-free treatment compared to 151 ± 24.8 nodules g^{-1} of root dry weight in the weedy treatment (data not shown). This represented approximately a 42% reduction in total nodule number per plant at the unifoliate stage when

Table 4. The effect of aboveground neighboring weeds on root volume (cm^3), average root diameter (mm) and number of root tips measured from soybean emergence until the second-trifoliate stage of soybean development. Data are means (± SE). Type I error rate set at 5% significance level.

		Root volume			Average root diameter			Number of root tips		
		Treatment			Treatment			Treatment		
DAP	Soybean stage	Weed free	Weedy	P value	Weed free	Weedy	P value	Weed free	Weedy	P value
		cm^3			mm					
6	VE	0.10 (0.094)	0.10 (0.096)	0.97	1.08 (0.021)	1.05 (0.022)	0.26	31 (31.9)	34 (32.9)	0.93
8	VC	0.38 (0.090)	0.36 (0.090)	0.80	0.81 (0.019)	0.78 (0.019)	0.23	105 (29.6)	82 (29.8)	0.48
12	V1	0.93 (0.089)	0.88 (0.090)	0.53	0.70 (0.019)	0.71 (0.019)	0.52	245 (29.6)	243 (30.0)	0.96
17	V2	2.05 (0.095)	1.80 (0.094)	0.006	0.61 (0.021)	0.58 (0.021)	0.45	732 (32.2)	683 (31.8)	0.17
21	V3	2.65 (0.090)	2.20 (0.090)	< 0.0001	0.56 (0.019)	0.53 (0.019)	0.28	981 (29.8)	965 (29.8)	0.60

[a] Abbreviations: DAP, days after planting; VE, emergence; VC, cotyledon; V1, unifoliate; V2, first trifoliate; V3, second trifoliate.

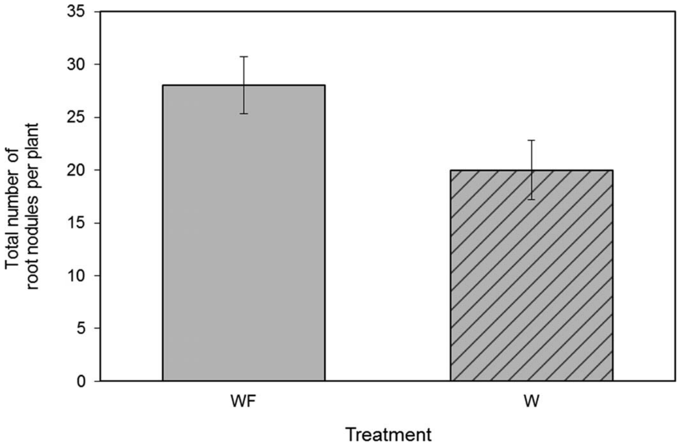

Figure 1. Total number of root nodules per unifoliate seedling as influenced by the aboveground neighboring weeds. WF and W refer to soybean seedlings grown under weed-free and weedy conditions, respectively.

soybean seedlings were grown in the presence of above ground neighboring weeds. Despite this difference in nodule number, at this stage of development no difference in total plant carbon or nitrogen content was detected between treatments (data not shown). This reduction or possible delay in nodule development may have occurred as a result of physiological changes within the root system in response to exposure to low R : FR reflected from aboveground neighboring weeds.

The Presence of Aboveground Neighboring Weeds Increased H_2O_2 Content and Lipid Peroxidation in Soybean Seedling Roots. Low R : FR reflected from aboveground neighboring weeds resulted in an increase in H_2O_2 and MDA content in root tissue of unifoliate soybean seedlings (Figures 2 and 3). The H_2O_2 content increased from 0.54 nM g^{-1} FW ± 0.023 in the weed-free treatment, compared to 0.72 nM g^{-1} FW ± 0.023 in the weedy treatment (Figure 2). A similar response was observed for MDA content (Figure 3). In the weed-free treatment, MDA content of the root tissue was 32.8 nM g^{-1} FW ± 1.45 vs. 41.4 nM g^{-1} FW ± 1.45 in the weedy treatment.

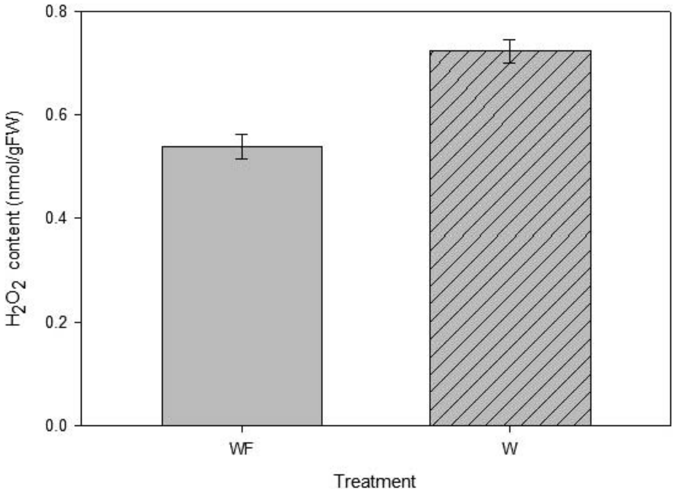

Figure 2. Hydrogen peroxide (H_2O_2) content in the root tissue of unifoliate soybean seedlings as influenced by the aboveground neighboring weeds. WF and W refer to soybean seedlings grown under weed-free and weedy conditions, respectively.

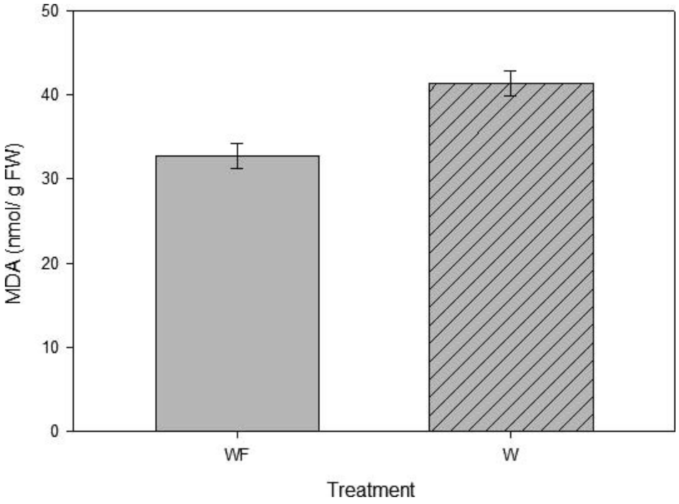

Figure 3. Malondialdehyde (MDA) content in root tissue of soybean seedlings as influenced by the aboveground neighboring weeds at the unifoliate stage of soybean development. WF and W refer to soybean seedlings grown under weed-free and weedy conditions, respectively.

The Presence of Aboveground Neighboring Weeds Decreased Flavonoid Content and DPPH-Radicle Scavenging Activity in Soybean Seedling Roots. Root flavonoid content and DPPH-radicle scavenging activity were lower in unifoliate soybean seedlings exposed to aboveground neighboring weeds, compared to soybean seedlings grown in a weed-free environment (Figures 4 and 5). Total flavonoid content in the weed-free treatment was 3.18 mg g^{-1} FW ± 0.122, compared to 2.38 mg g^{-1} FW ± 0.122 in the weedy treatment. In addition, this decline in flavonoid content was accompanied by a similar decrease in DPPH-radicle scavenging activity. The DPPH-radicle scavenging activity in the root tissue was reduced from 33.07% of control ± 0.743 in the weed-free treatment to 21.15% of control ± 0.743 in the weedy treatment.

In order to explore the molecular mechanisms contributing to this finding, a quantitative real-time PCR was conducted to investigate the transcription level of *Gm*IFS, a key gene involved in flavonoid biosynthesis (Yoo et al. 2013), and *Gm*N93, a key

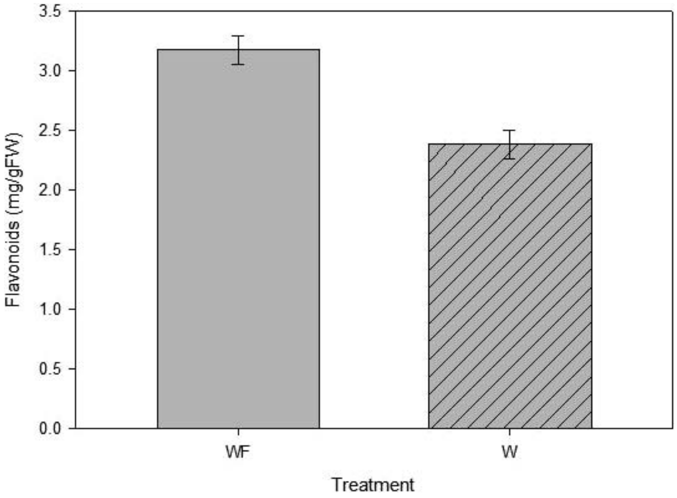

Figure 4. Flavonoid content in in root tissue of soybean seedlings as influenced by the aboveground neighboring weeds at the unifoliate stage of soybean development. WF and W refer to soybean seedlings grown under weed-free and weedy conditions, respectively.

gene involved in nodule formation (Reddy et al. 1998). The presence of neighboring weeds caused a significant reduction in the expression of the *Gm*IFS gene, and of the *Gm*N93 gene (Figure 6). For example, *Gm*IFS gene expression was reduced to more than half-fold (0.43-fold) in soybean seedlings growing under weedy conditions, compared to those under weed-free conditions. This inhibition of *Gm*IFS gene expression would contribute to the observed reduction of total flavonoid content under weedy conditions. In addition, the *Gm*N93 transcription level was reduced to 0.49-fold under the weedy treatment vs. 1-fold under weed free treatment. This gene inhibition may account for the lower number of nodules formed on soybean roots growing in the presence of neighboring weeds and exposed to low R : FR signal.

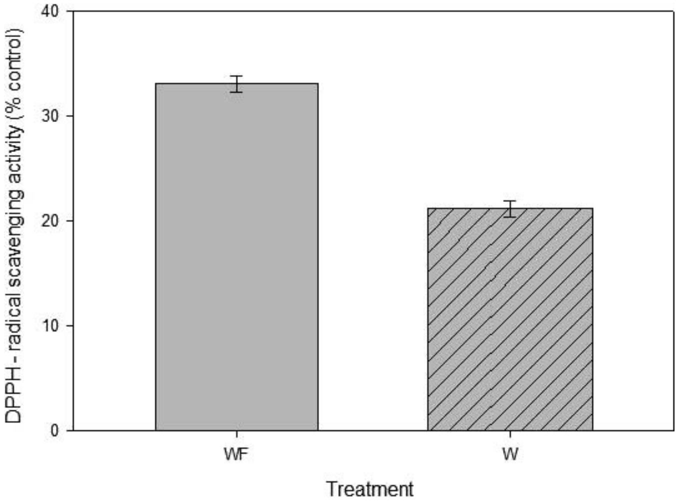

Figure 5. 1,1-diphenyl-2-picrylhydrazyl (DPPH) –radicle scavenging activity in the root tissue of soybean seedlings as influenced by the aboveground neighboring weeds at the unifoliate stage of soybean development. WF and W refer to soybean grown under weed-free and weedy conditions, respectively.

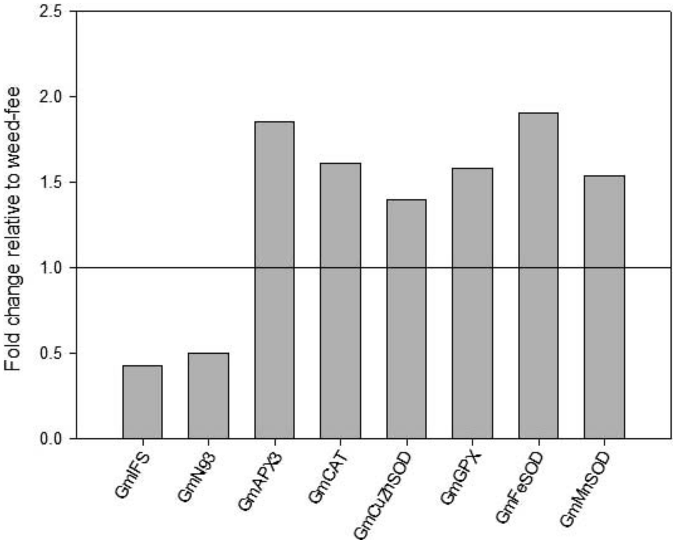

Figure 6. QRT-PCR analysis of the transcripts level of isoflavone synthase (*Gm*IFS), an early nodulin gene (*Gm*N93), ascorbate peroxidase (*Gm*APX3), catalase (*Gm*CAT), copper-zinc superoxide dismutase (*Gm*CuZnSOD), glutathione peroxidase (*Gm*GPX), iron superoxide dismutase (*Gm*FeSOD), and manganese superoxide dismutase (*Gm*MnSOD) as influenced by the presence of aboveground neighboring weeds at the unifoliate stage of soybean development. Data presented relative to weed-free treatment.

The Presence of Aboveground Neighboring Weeds Regulated the Transcription of the Scavenging Enzymes Genes in Soybean Roots. Qualitative real-time PCR was conducted to test the effect of neighboring weeds on the transcript levels of *Gm* APX3, *Gm*CAT, *Gm*CuZnSOD, *Gm*GPX, *Gm* Fe SOD, and *Gm*MnSOD. These six common genes are known to encode for the production of scavenging enzymes. Interestingly, the transcript level of all six genes increased in the root tissue of soybean seedlings exposed to low R : FR, compared with the weed-free control (Figure 6). The transcript levels for the six genes, *Gm*APX3, *Gm*CAT, *Gm*CuZnSOD, *Gm* GPX, *Gm*FeSOD, and *Gm*MnSOD, were 1.86-, 1.61- 1.40-, 1.58-, 1.91-, and 1.54-fold higher, respectively, in seedlings exposed to low R : FR relative to seedlings kept weed-free.

Under conditions of nonlimiting resources, low R : FR reflected from aboveground neighboring weeds reduced soybean seedling root biomass, length, surface area, and volume. Reductions in these root parameters were first evident in root surface area and volume at the first trifoliate leaf stage. Reductions in root length were detected by the second trifoliate leaf stage. A similar reduction in root biomass attributed to low R : FR has been reported in several studies (Afifi and Swanton 2011; Kasperbauer and Karlen 1994; Liu et al. 2009; Page et al. 2009; Pecháčková 1999; Skálová and Vosátka 1998; Sparkes et al. 2008). For example, Sparkes et al. (2008) observed a reduction in the root volume of wheat under low R : FR even though light quantity (PPFD) was held constant. Afifi and Swanton (2011) also found a reduction in total root volume, surface area, and biomass of maize seedlings when exposed to low R : FR reflected from aboveground neighboring weeds.

Prior to this study, limited work has been done to understand how soybean root morphology changes in response to low R : FR reflected from neighboring weeds. For example, Green-Tracewicz et al. (2011, 2012) conducted two studies on the expression of the shade-avoidance response in soybean plants as a consequence of weed competition. This previous research established clearly that FR light had a significant negative influence on soybean root biomass. These researchers did not study, however, the influence of FR light on specific root morphological parameters, such as length, surface area, and volume. The reduction found in root morphological parameters in the present study would explain the loss in total root biomass reported in this and previous studies (Green-Tracewicz et al. 2011, 2012). Results from the present study indicate that changes in root growth and morphology can be influenced negatively very early in soybean seedling development. Soybean roots are responsible for providing anchorage and uptake of water and nutrients; therefore, any alteration in length, surface area, volume, and/or biomass will reduce the seedling's ability to explore for and capture soil nutrients. In addition, these early changes may limit the ability of soybean seedlings to respond appropriately to subsequent abiotic and biotic stresses, thereby reducing yield potential when grown under field conditions.

In this study, soybean seedlings continuously exposed to aboveground neighboring weeds exhibited a reduction in nodule number per plant. The downregulation of the *GmN93* gene would, in part, account for this reduction. Several previous studies have examined the effects of FR light on legume nodulation (Balatti and Montaldi 1986; Kasperbauer et al. 1984; Kasperbauer and Hunt 1994; Lie 1969; Sheehy et al. 1983). These studies examined pulses of supplemental FR on various crop plants, including pea and soybean. They too concluded that a reduction in R : FR reduced legume root nodule numbers.

In addition to the reduction in nodule number, the roots of soybean seedlings also exhibited a reduction in total flavonoid content, relative to those seedlings kept weed-free. Flavonoids serve two essential roles in soybean seedlings: (1) acting as signal molecules to symbiotic microbes, and (2) acting as a nonenzymatic reactive oxygen species (ROS) scavenging mechanism (Subramanian et al. 2006, 2007; Taylor and Grotewold 2005). It has been well established that several flavonoids exuded from plant roots act as signal molecules inducing the transcription of bacterial genes, initiating the infection process (Treutter 2006). This is a significant role that flavonoids play in improving plant growth and fitness (Treutter 2006). The reduction in total root flavonoid content observed in soybean seedlings exposed to aboveground weeds would invariably contribute to the reduction in nodule number per plant. The seedling's ability to communicate with the rhizobia bacteria in the soil and establish a symbiotic relationship would be affected negatively. In addition, this reduction in nodule number per plant could also lead to a nitrogen deficiency, which may account for an initial reduction in soybean growth observed in previous studies (Green-Tracewicz et al. 2011, 2012).

Exposure to low R : FR conditions triggered the accumulation of H_2O_2 and subsequent lipid peroxidation of cell membranes in root tissue of soybean seedlings at the unifoliate stage of development. Hydrogen peroxide is a well-known ROS, which has been observed to increase under conditions of biotic and abiotic stress (Gill and Tuteja 2010). Interestingly, under growth conditions in which all resources (light, water, and nutrients) were supplied in sufficient quantities in order to eliminate direct competition, the production of H_2O_2 was triggered by the low R : FR signal. As with the rapid changes in root growth and morphology, changes in H_2O_2 content were detected very early in soybean development. Similarly, Afifi and Swanton (2012) and Afifi et al. (2014) reported accumulation of H_2O_2 in the first leaf and crown root tissue of maize seedlings exposed to the presence of aboveground neighboring weeds. The accumulation of H_2O_2 in the roots of soybean seedlings could result in major cellular damage, such as DNA alterations, oxidation of proteins, and lipid peroxidation (Gill and Tuteja 2010).

Levels of lipid peroxidation have been used widely as an indicator of ROS-mediated damage to cell membranes under stress conditions (Tanou et al. 2009). Recently, Afifi et al. (2014) found an increase in MDA content in the first leaf and crown root tissue of maize seedlings exposed to low R : FR as a consequence of the presence of aboveground neighboring weeds. The buildup of H_2O_2 and the subsequent cellular damage could reduce soybean seedling vigor and invariably reduce the ability of soybean seedlings to respond to further biotic and abiotic stress.

In this study, the transcript level of *Gm*APX3, *Gm*CAT, G*m*CuZnSOD, G*m*GPX, *Gm*FeSOD, and *Gm*MnSOD in the roots of unifoliate soybean seedlings were found to increase under low R : FR conditions. Similar results were found in maize by Afifi and Swanton (2012) and Afifi et al. (2014). Enhanced production of ROS during stress can damage cells; however, ROS is also thought to act as a signal for the activation of stress-response and defense pathways of scavenging enzymes (Gill and Tuteja 2010). The accumulation of H_2O_2 in the root tissue of unifoliate soybean seedlings exposed to the aboveground low R : FR is consistent with an increase in the transcript levels of *Gm*APX3, *Gm*CAT, *Gm*CuZnSOD, *Gm*GPX, *Gm*FeSOD, and *Gm*MnSOD.

Plants have several mechanisms that can reduce H_2O_2 production during a stressful period. These mechanisms include anatomical adaptations and physiological and molecular changes (Mittler 2002). Plants with the ability to scavenge and/or control the level of cellular H_2O_2 will be better adapted to survive (Gill and Tuteja 2010). Efficient scavenging of ROS requires the action of both enzymatic and nonenzymatic scavenging mechanisms (Sharma et al. 2012). The enzymatic mechanism includes enzymes such as ascorbate peroxidase (APX), catalase (CAT), various forms of superoxide dismutase (SOD), and glutathione peroxidise (GPX). Nonenzymatic compounds, such as ascorbic acid, phenols, flavonoids, and anthocyanins, are known to be involved in antioxidant defence systems (Treutter 2006). Polyphenols, such as flavonoids, can chelate transition metal ions, directly scavenge ROS, delay diffusion of free radicles, limit peroxidative reactions, and inhibit lipid peroxidation (Sharma et al. 2012).

A reduction in both flavonoid content and DPPH-radicle scavenging activity in root tissue of unifoliate soybean seedlings were observed in this study. This reduction in flavonoid content may be attributed to the observed downregulation of *Gm*IFS. Similar results were reported by Afifi et al. (2014), who investigated the effects of aboveground neighboring weeds on the total phenolic content, of which flavonoids are a component, and DPPH-radicle scavenging activity of maize seedlings. It was found that the presence of neighboring weeds reduced total phenolic content in the first leaf, stem, and crown roots of maize seedlings (Afifi et al. 2014). An identical response was observed in the DPPH-radicle scavenging activity in the same tissues (Afifi et al. 2014). Thus, the reduction in soybean root flavonoid content and DPPH-radicle scavenging activity observed in soybean seedlings exposed to neighboring weeds is indicative of a decline in the plant's ability to scavenge for ROS nonenzymatically. This decline increases the potential for these molecules to cause damage, potentially decreasing the ability of the seedling to deal with subsequent abiotic and biotic stresses.

Abiotic and biotic stresses, including insect and disease infestation, have been reported to influence the flavonoid content in various crops (Treutter 2006). Both frost hardiness and drought tolerance have been attributed to "flavonoids or other phenolic compounds with respect to functions in the cell wall and membranes" (Tattini et al. 2004; Treutter 2006). It has also been reported that flavonoids may play a role in tolerance to toxic metals, such as aluminum (Barceló and Poschenrieder 2002). Ryan et al. (2002) found that flavonoids play a predominant role in photoprotection in *Petunia* leaves. The present study, however, is the first to report a reduction in root flavonoid content of unifoliate soybean seedlings caused by a low R : FR signal reflected from aboveground neighboring weeds.

Early physiological mechanisms of nonlimiting resource weed competition were explored in this study. These mechanisms occurred in soybean seedlings primarily in response to the detection of the R : FR signal reflected from aboveground neighboring weeds. The detection of low R : FR triggered a series of physiological changes that occurred very early in the development of a soybean seedling. It is hypothesized that these changes may result in a physiological cost, which limits the soybean plant's ability to respond to subsequent abiotic and biotic stresses that will occur under field conditions. Studying interactions between aboveground signals and belowground plant responses will invariably improve our understanding of the mechanisms of early plant competition.

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